

**BRIEF REPORT**

**Online Supplement**

**Methods and Materials**

**Smooth Muscle Cells Derived from Second Heart Field and Cardiac Neural Crest  
Reside in Spatially Distinct Domains in the Media of the Ascending Aorta**

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## Methods and Materials

### Mice and study design

Female ROSA26R<sup>LacZ</sup> (#003474) and Wnt1-Cre (#022501) mice were purchased from The Jackson Laboratory. Mef2c-Cre mice (#030262) were purchased from the Mutant Mouse Resource and Research Center. Either Wnt1- or Mef2c-Cre male mice were bred to ROSA26R<sup>LacZ</sup> female mice to trace CNC- or SHF-derived cells, respectively. Mice were fed a normal laboratory rodent diet and provided with drinking water from a reverse osmosis system. The room light:dark cycle was 14:10 hr. All procedures were approved by the University of Kentucky's IACUC. Histological analysis was performed using whole (n = 3 - 4) and sectioned tissues (n = 6).

### Tissue

Tissues were harvested from mice at 3, 12, and 25 weeks of age. At study termination, mice were euthanized using a ketamine/xylazine cocktail (90 mg/kg, 10 mg/kg, respectively). The thoracic cavity was opened and saline was perfused through the left ventricle. Hearts and thoracic aortas were dissected free, and placed in paraformaldehyde (4% wt/vol) for gross tissue histology or a block of OCT for frozen sectioning. For frozen sectioning, serial cross-sections (10  $\mu$ m) were collected starting at the aortic valves and ending at the innominate artery. Two to three serial series of 10 slides with 9 sections per slide were obtained per ascending aorta. Sagittal sections (10  $\mu$ m) which included the whole ascending aorta were also collected.

### Detection of $\beta$ -galactosidase activity

Whole and sectioned tissues were incubated with X-gal (V3941, Promega) and eosin B (E8764, Sigma). Briefly, tissues were fixed in paraformaldehyde (4% wt/vol), and then preincubated in buffer containing sodium phosphate (100 mM, pH 7.3), MgCl<sub>2</sub> (2 mM), sodium deoxycholate (0.01% wt/vol) and NP40 (0.02% wt/vol). X-gal (1 mg/ml), potassium ferricyanide (5 mM), and potassium ferrocyanide (5 mM) were added to buffer and samples were incubated overnight at room temperature. Whole tissues were post-fixed with formalin. Tissue sections on slides were rinsed free of X-gal, and then incubated with eosin (1% wt/vol) for 2 minutes, and cover slipped.

### Immunofluorescent staining

For immunofluorescence studies, tissue sections (10  $\mu$ m) were incubated with paraformaldehyde (4% wt/vol) for 1 hour at room temperature. Sections were subsequently incubated with goat serum for 1 hour at 40°C. The following antibodies were used for primary antibodies -  $\beta$ -galactosidase (ab9361, Abcam, 1  $\mu$ g/ml),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, ab5694, Abcam, 2  $\mu$ g/ml), CD31 (ab7368, Abcam, 10  $\mu$ g/ml), and ER-TR7 (ab51824, Abcam, 2  $\mu$ g/ml). Detection of primary antibodies was visualized with a goat anti chicken IgY antibody (Alexa Fluor 488, ab150169, Abcam, 3.9  $\mu$ g/ml), goat anti rabbit IgG (Alexa Fluor 568, ab175471, Abcam, 4  $\mu$ g/ml) or goat anti rat IgG (Alexa Fluor 568, ab175710, Abcam, 4  $\mu$ g/ml), respectively. Slides were cover slipped with Fluorshield Mounting Media with DAPI (ab104140, abcam). Confocal images were obtained with a Nikon confocal microscope A1RSi and processed with NIS-Elements AR software.

## **Histogram**

To assess the medial distribution of  $\beta$ -gal activity, histograms were made using 40x highly magnified images of X-gal staining by NIS-Elements AR software. Briefly, 40x magnified images were captured from the middle of the ascending aorta. White color was subtracted from the 40x image and then the image was visualized through the green filter to emphasize positive areas of X-gal staining. Then a histogram was made from an entire image. Lines for internal and external elastin lamina were drawn using autofluorescence to detect the elastin fibers (Figure I in online-only Data Supplement). Reproducibility of histogram data were determined using three mice per genotype.